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PRINCIPAL INVESTIGATOR: Katherine A. Hoadley, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina at Chapel Hill
Chapel Hill, NC 27599

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Katherine A. Hoadley, Ph.D.

Email: hoadley@med.unc.edu

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14. ABSTRACT The basal-like subtype of breast cancer is both estrogen receptor and HER2 negative and therefore is not effectively treated by hormonal therapy or trastuzamab. The purpose of this research is to identify treatment options for this subset of breast cancer patients. Breast cell lines of basal-like and luminal origin were treated with five different chemotherapeutics to determine sensitivity levels. The basal-like cell lines were more sensitive to carboplatin than luminal lines. Next, we focused on identifying a biologic therapy targeting the basal-like subtype. HER1/EGFR is expressed in approximately 50% of the basal-like tumors while not expressed in the luminal tumors. The basal-like cell lines showed an increased sensitivity to several EGFR inhibitors compared to the luminal lines. Combinations of two EGFR were synergistic with carboplatin. This work is support for a clinical trial at UNC, which will treat basal-like breast cancer patients with a HER1 inhibitor with or without carboplatin. An EGFR activation signature was identified and shown to predict poor outcome in two breast tumor data sets. In basal-like tumors, there may be many mechanisms for activation of this pathway but many appear to be downstream of EGFR in the RAS-MEK-ERK pathway. This work shows that basal-like may need to be further stratified to identify successful treatment regimens.

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Table of Contents

Cover……1

SF 298……..……2

Introduction……..…….……...4

Body……4

Key Research Accomplishments…….……6

Reportable Outcomes……6

Conclusions…….……..7

References……..…8

Appendices…….…8

Introduction

Both clinical data and gene expression data have demonstrated that there is more than just one type of breast cancer. Gene expression data identified five subtypes of breast cancer: the estrogen receptor positive Luminal A and B, HER2+, normal-like and the basal-like (*1*). The basal-like subtype, which accounts for 10-15% of tumors, is negative for both the estrogen receptor and HER2 and is therefore not a candidate for hormonal therapy or trastuzumab. Most of the work in understanding chemotherapeutic sensitivity has been accomplished with the luminal subtypes, which are more prevalent and have better outcomes. This prompted the work, demonstrated here, to identify chemotherapeutics with increased sensitivity and identify biologics that specifically target markers of the basal-like subtype. One candidate is the HER1/EGF receptor, which is highly expressed in about 50% of breast cancers (*2*). Many inhibitors to this receptor are available and are in use in a variety of other cancers, decreasing the potential time to clinical use. This work describes the identification of a potential targeted chemotherapy and the evaluation of the EGFR signaling pathway in the breast cancer subtypes.

Body

Task 1: Identify differences in toxicant sensitivity and gene expression profiles between basal and luminal breast derived cell lines treated with a diverse set of toxicants.

As stated in the April 2006 Annual Summary, comparison of our breast cell lines to a panel of chemotherapeutic agents identified the two basal-like tumor derived cell lines as slightly more resistant to 5-fluorouracil and more sensitive to carboplatin compared to the HMEC and luminal cell lines. Using *Significance Analysis of Microarrays*, I compared the two tumor-derived basal lines treated with carboplatin with the normal HMEC basal-like lines treated with carboplatin. There were many dynamic changes in gene expression in the HMEC lines while the SUM lines were relatively unchanged for these genes (data not shown). Unfortunately, many of the probes were ESTs or hypothetical proteins. Gene Ontology analysis did not identify any significant functional groups of genes that were enriched in the gene list. The expression patterns, while not identifiable at the current stage, may have resulted from the SUM lines inability to fix the carboplatin-induced damage. BRCA1 has been demonstrated to be necessary for the recruitment of RAD51 to sites of platinum-induced damage (*3-5*). The SUM149 line has a mutant *BRCA1* and SUM102 have reduced transcript levels of *BRCA1* (*6*). BRCA1 mutant phenotypes have been associated with predominately the Basal-like phenotype (*7-12*). Therefore, carboplatin or other platinum drugs could, in essence, behave like a targeted chemotherapy for the treatment of basal-like cancers.

Task 2. Evaluate the sensitivity of basal-like and luminal breast cell lines to HER1/EGFR inhibitors.

Chemosensitivity to two additional EGFR inhibitors, erlotinib and cetuximab, were evaluated on my panel of breast cancer cell lines. While sensitivity in my panel of cell lines was more variable to these EGFR inhibitors compared to gefitinib, where the basal-like lines were 2-100 fold more sensitive than the luminal lines, all of the cell lines sensitive to these agenets were of the basal-

like subtype (Table 1). The SUM102 line was the only cell line sensitive to all three EGFR inhibitors and the only one sensitive to cetuximab (dose range 0-100μg/mL).

Microarray analysis was performed to evaluate the EGFR activation signature in the SUM102 cell line. Cells were treated for 48h with either gefitinib or cetuximab to inhibit the signaling pathway. Media with inhibitor was replaced with fresh media without inhibitor and time points were taken at 4h, 8h, and 24h. Unsupervised analysis of the data showed that the two inhibitors had high similarity in their gene expression alterations (Figure 2). To view the EGFR activation signature on breast tumors, a one class *Significance Analysis of Microarrays* was used to identify the top 500 induced genes from the 4h, 8h, and 24h time points. These genes were clustered on a UNC tumor data set of 248 tumors representing all five intrinsic breast cancer subtypes. Three distinct gene clusters (criteria: >20 genes, Pearson node correlation > 0.55) were observed across the tumor data (Figure 3). The first cluster was high in a mixed tumor group, the second was high in mostly basal-like tumors, and the third was high in basal-like, HER2+/ER-, and luminal tumors. These three clusters were evaluated on an independent NKI data set of 295 breast tumors. High expression of each cluster was correlated with poor outcome (Figure 4). Chi-square analysis identified correlations of each cluster with subtype (Table 3). High expression of each cluster was associated with most of the tumors of the basal-like subtype and approximately half of the HER2+/ER- and luminal B tumors. While only 50% of basal-like tumors have high expression of EGFR protein, greater than 95% of the basal-like subtype had high expression of at least one, if not all three, of these clusters indicating a dependence on this pathway for growth.

Since EGFR is not high in all basal-like cells, I examined expression of other genes in the pathway and correlated them with both subtype and cluster (Figure 3, Tables 3 and 4). High expression of many of the genes in this pathway was correlated with different subtypes or with different clusters. This really demonstrates that each of the subtypes uses the EGFR signaling pathway differently. More details about the correlations are discussed in the appended manuscript. High expression of many genes downstream of EGFR was associated with the basal-like subtype, many of which have been suggested to confer EGFR-independent growth. Only about 10% of the basal-like tumors had high expression of EGFR and ligands with low expression of these downstream genes, suggesting that direct inhibition of EGFR may not be effective in many basal-like tumors.

While looking at gene expression ordered by hierarchical clustering can be informative, I also wanted to observe these genes in the sense of a pathway. I built an EGFR pathway in Cytoscape based on several pathway databases and reviews. The gene expression of each subtype or of individual tumors were uploaded and then viewed within the context of the pathway. Many differences were observed across the tumor subtypes showing characteristic differences in how each subtype utilizes the pathway (Figure 5). The luminal A subtype had low expression of the pathway while luminal B had higher expression of some of the genes and high expression of *HRAS* was correlated with approximate half of the luminal B tumors. The HER2+/ER- tumors, as expected, had high expression of *HER2* as well as other genes including *HRAS*. The basal-like subtype had high expression of most of the genes in the pathway; however, the mechanism of pathway activation varied from tumor to tumor (Figure 6). The treatment options for basal-like patients will be complex and may require inhibition at multiple different steps. Many of the tumors were high for *CRYAB*, *MEK*, and the *KRAS* amplicon all of which signal through the

RAS-MEK-ERK pathway. MEK inhibitors are currently in clinical trials and may be more beneficial than the EGFR inhibitors for the basal-like subtype.

Task 3. Test combination therapies of HER1/EGFR inhibitors and a diverse set of toxicants

Previously, I demonstrated combination analysis of the four tumor-derived breast cell lines with a single concurrent treatment of gefitinib and chemotherapy. A basal-like clinical trial in process at UNC is examining cetuximab and carboplatin. Therefore, we examined cetuximab in combination with carboplatin as well as doxorubicin, 5-fluorouracil, and paclitaxel in the tumor-derived basal-like cell line SUM102, the only line sensitive to cetuximab. Full methods and data are included in the appended manuscript. Many studies have shown that the order of the drugs can have various effects on cell growth (*13, 14*). I evaluated sequence dependent effects of four treatments: (1) 72h cetuximab followed by 72h chemotherapy, (2) 72h chemotherapy followed by 72h cetuximab, (3) 72h chemotherapy plus cetuximab, and (4) 144h chemotherapy plus cetuximab. In general, chemotherapy first or the 144h concurrent combination were more growth inhibitory than the short-term concurrent combination or cetuximab first. Cetuximab combinations with carboplatin were the most growth inhibitory and had the highest levels of synergistic interaction between the two drugs for the carboplatin first or the long-term concurrent. This is very promising for the cetuximab clinical trial. Combinations with 5-fluorouracil were similar, while combinations with doxorubicin required higher dose levels to achieve synergy. Only combinations with paclitaxel were antagonistic at all doses (Figure 1, appended manuscript). However, SUM102 cells were extremely sensitive to paclitaxel as a single agent and the addition of cetuximab may appear antagonistic because of the already high sensitivity observed (Table 2, appended manuscript).

Key research accomplishments

- Basal-like tumor derived lines are more sensitive to carboplatin
- Basal-like cells lines are sensitive to gefitinib
- Combination of carboplatin and gefitinib was synergistic in vitro
- High expression of EGFR activation signatures is correlated with poor outcomes
- Basal-like subtype has high expression of genes that may confer EGFR-independent growth
- Basal-like subtype has many different mechanism of EGFR pathway activation

Reportable outcomes

Speaker Abstract	Toxicogenomics 2006 Bi-annual Meeting
Manuscript	EGFR Signaling Varies with Breast Tumor Subtype Submitted to Breast Cancer Research December 2006
Ph.D.	Genetics and Molecular Biology Awarded December 2006

Conclusions

The breast cancer subtypes have been robustly identified in many different studies regardless of differences in demographics of the data set, sample collection, preparation, or platform (*1, 11, 15-17*). Thus, the inherent differences across the subtypes imply that each subtype will require a different and possibly unique treatment approach. In the case of ER+ or HER2+/ER- tumors, there are several targeted therapies that have shown high effectiveness in many of the patients with these tumors. However, the basal-like subtype, which has poor outcomes, lacks both of these markers and few have examined how they respond to chemotherapies. The research described here specifically examined cells lines and tumors of basal-like origin to identify chemotherapy or targeted therapy options for treatment.

The basal-like cell lines were more sensitive to carboplatin compared to the normal HME basal-like lines and the luminal cell lines. This may be a result of the low to absent functional BRCA1 in the SUM lines (*6*). Mutant or low BRCA1 is associated with the basal-like phenotype (*7-12*). Since BRCA1 is required for the repair of DNA damage (*3-5*), platinum-based chemotherapy treatments may have an increased benefit for the basal-like subtype. There are two clinical trials – [http://www.clinicaltrials.gov/ct/show/NCT00232505] and [http://www.clinicaltrials.gov/ct/show/NCT00248287] – where we will be able to test the benefit of carboplatin in basal-like patients.

EGFR protein expression is observed in about 50% of basal-like tumors (*2*). Many EGFR inhibitors have shown promise in other cancers. Therefore, EGFR represented a good candidate for a targeted therapy for basal-like tumors. In our cell line model, cell lines that were sensitive to any of our three EGFR inhibitors were of basal-like subtype. I also demonstrated that the combination of two EGFR inhibitors, cetuximab and gefitinib, were synergistic with carboplatin in combination treatments.

Using microarrays, I identified an EGFR pathway activation signature in hopes of being able to identify tumors that may benefit from EGFR inhibitors. High expression of genes in the activation cluster was predictive of poor outcome. Further analysis identified high expression of many genes downstream of EGFR that may confer EGFR-independent activation of the pathway were highly correlated with the subtype. Many of these genes are in the RAS-MEK-ERK pathway. While only 50% of tumors express EGFR protein, up to 95% of the tumors show high activation of genes within this pathway. The basal-like tumors, themselves, were very heterogeneous with many different potential EGFR pathway activations. This data strongly supports focusing targeted therapies farther downstream of EGFR such as the newer MEK inhibitors. In the case of the basal-like tumors, multiple inhibitors and chemotherapy combinations may be required to effectively treat this subtype.

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Appendices

1. Manuscript: EGFR Signaling Varies with Breast Tumor Subtype (Contains Referenced Figures/Tables)
2. Toxicogenomics 2006 Bi-Annual Meeting Speaker Abstract
3. CV

EGFR signaling varies with breast tumor subtype

Katherine A. Hoadley[1-3], Victor J. Weigman[2-4], Cheng Fan[2,3], Lynda R. Sawyer[5], Xiaping He[2,3], Melissa A. Troester[6], Carolyn I. Sartor[3,7], Thais Rieger-House[8], Philip S. Bernard[8], Lisa A. Carey[5], and Charles M. Perou[1-3,9,*]

[1]Curriculum in Genetics and Molecular Biology, [2]Department of Genetics, [3]Lineberger Comprehensive Cancer Center, [4]Department of Biology, Program of in Bioinformatics and Computational Biology, [5]Division of Hematology/Oncology, Department of Medicine, [7]Department of Radiation Oncology, [9]Department of Pathology & Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC

[6]Department of Public Health - Biostatistics and Epidemiology Concentration, University of Massachusetts Amherst, Amherst, MA

[8]Huntsman Cancer Institute and Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT

[*]Corresponding Author:
Charles M. Perou
Lineberger Comprehensive Cancer Center
CB# 7295
The University of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7295
Email: cperou@med.unc.edu
Phone: (919) 843-5740
Fax: (919) 843-5718

Running Title: EGFR signaling in breast cancer
Keywords: breast cancer, chemotherapy, EGFR, HER1, microarray

All figures and text can be obtained at https://genome.unc.edu/cgi-bin/SMD/publication/viewPublication.pl?pub_no=62

Author email addresses
Katherine A. Hoadley: hoadley@med.unc.edu
Victor J. Weigman: victor@med.unc.edu
Cheng Fan: cfan2004@gmail.com
Lisa A. Carey: lisa_carey@med.unc.edu
Lynda R. Sawyer: lrsawyer@med.unc.edu
Xiaping He: xiaping@med.unc.edu
Melissa A.Troester: troester@schoolph.umass.edu
Carolyn I. Sartor: carolyn_sartor@med.unc.edu
Thais Rieger-House: thais.riegerhouse@hci.utah.edu
Philip S. Bernard: phil.bernard@hci.utah.edu
Charles M. Perou: cperou@med.unc.edu

ABSTRACT

Introduction

The epidermal growth factor receptor (EGFR/HER1) and its downstream signaling events are important for regulating cell growth and behavior in many epithelial tumors including lung and colon. In breast cancers, the role of EGFR is complex and may vary with estrogen receptor (ER) status.

Methods

To investigate the role of EGFR signaling in breast cancer, several breast basal-like and luminal epithelial cell lines were examined for sensitivity to the EGFR inhibitors gefitinib and cetuximab. We identified an EGFR-activation profile in the basal-like breast cancer cell line SUM102 and analyzed expression of these genes in human breast tumors.

Results

The breast basal-like cell lines were more sensitive to gefitinib compared to the luminal lines. The basal-like cell line SUM102 was the only cell line sensitive to cetuximab. The basal-like tumor derived lines were also the most sensitive to carboplatin, which acted synergistically with cetuximab. Using SUM102 cells, we identified an EGFR-activation profile that included a strong MEK-dependent signature. The EGFR-activation signature was next used to analyze a large panel of primary breast tumors. Three distinct clusters of genes were evident *in vivo*, two of which were strongly predictive of poor patient outcomes. These two poor prognostic signatures were highly expressed in most basal-like tumors and in approximately half of the HER2+/ER- and Luminal B tumors. Ninety percent of the basal-like tumors that showed high expression the EGFR-activation profiles also showed high expression of *CRYAB* and/or a *KRAS*-amplicon signature.

Conclusion

These data suggest that most basal-like tumors have an EGFR-activation profile, however, few may respond to the direct inhibition of EGFR due to ligand independent activation of the EGFR-RAS-MEK pathway via *CRYAB* or *KRAS* function. Thus, for those tumors that show a ligand independent EGFR-activation profile, alternative strategies that target downstream components like MEK may prove to be viable alternatives.

Introduction

The epidermal growth factor receptor (EGFR/HER1) is a member of the human epidermal growth factor receptor (HER) family of transmembrane receptor tyrosine kinases that is linked to growth control, cell adhesion, mobility, and apoptosis [1]. As such, EGFR is an important regulator of epithelial cell biology, but its function in breast tumors may vary according to other clinical features like estrogen receptor (ER) status. Microarray studies have identified several subtypes of breast cancer arising from at least two different epithelial cell types [2-5]. Two of the molecular subtypes of breast cancer are partly defined by the expression of ER, while another is partly defined by the genomic DNA amplification and high expression of HER2 (i.e. HER2+/ER-, see [5]). The basal-like subtype has low expression of both ER and HER2, however, EGFR was found to be highly expressed in many of the basal-like tumors as assessed by both gene and protein expression [6].

EGFR overexpression has been reported in a variety of epithelial tumors [7], leading to the development of drugs directed against this receptor [8, 9]. One of these targeting strategies employs monoclonal antibodies (cetuximab) that bind the extracellular ligand-binding domain, while other strategies include small molecule inhibitors (gefitinib and erlotinib) that compete with ATP for binding to the intracellular tyrosine kinase domain [10-12]. In non-small cell lung cancer and breast cancer cell lines, it has been shown that some small molecule EGFR inhibitors increase cell killing when used in combination with chemotherapeutics [13, 14]; therefore, the interactions between EGFR inhibitors and cytotoxic agents represent a promising combination for the future treatment of epithelial tumors that are dependent upon EGFR-signaling.

The lack of clinical response in breast cancers to gefitinib *in vivo* has been partially attributed to activation of this pathway downstream of EGFR. Several studies have implicated the PI3K/AKT and MEK/ERK pathways as being responsible for EGFR inhibitor resistance. EGFR-independent activation of the PI3K/AKT pathway may occur through either loss of PTEN or mutation/activation of PI3K, both of which have been linked to gefitinib resistance [15-17]. Others have suggested that the MEK/ERK pathway may play a more important role in resistance to EGFR inhibitors [18-20]. Recently, Moyano *et al.* identified αB-Crystallin (CRYAB) as a protein that can constitutively activate the MEK/ERK pathway in breast cancer cells and caused this cell line to become EGF independent [21].

In this study, we used basal-like breast cell lines to examine the EGFR signaling pathway and its interactions with cytotoxic chemotherapeutics. Using an EGFR-activation profile derived from a basal-like tumor derived cell line, we determined that most basal-like and approximately 50% of Luminal B and HER2+/ER- tumors showed an EGFR-activation profile. EGFR-activation signatures may be useful in selecting patients for therapeutics that target the EGFR-RAS-MEK pathway.

Material and Methods

Cell Culture. SUM102 and SUM149 cells were a gift from Steve Ethier of Wayne State University [22] and represent cell lines derived from ER- and HER2- basal-like breast tumors. The SUM lines were maintained in an Epithelial Growth Medium developed by the Tissue Culture Facility at the University of North Carolina at Chapel Hill [23], and the SUM149 line

was further supplemented with 5% FBS. The MCF-7, ZR-75-1, HME-CC and ME16C cell lines were obtained and maintained as previously described [24, 25].

Cytotoxicity Assay. Cell line sensitivities to drugs were assessed using a mitochondrial dye conversion assay (MTT, Cell Titer 96, Promega) as described previously with the following modifications [24]. Cells were seeded into triplicate 96-well plates (SUM102, HME-CC, and ME16C – 5,000 cells/well, SUM149 – 10,000 cells/well, MCF-7 and ZR-75-1 – 7,000 cells/well) and allowed to adhere overnight. Cells were treated for 72h with a range of doses of individual drugs. Carboplatin, doxorubicin, 5-fluorouracil, paclitaxel, and LY294002 were purchased from Sigma. Gefitinib was a gift from AstraZeneca and cetuximab was purchased from the UNC Hospitals Pharmacy Storeroom. U0126 was purchased from Cell Signaling. The inhibitory concentration that caused a 50% reduction in MTT dye conversion (IC50) dose was determined as previously described [24].

Drug combination interactions were analyzed using methods developed by Chou and Talalay [26]. Using cell lines plated as described above, seven treatment combinations consisting of constant ratios of IC50 doses (ranging from one-eighth of each dose to eight times the IC50) were applied to cells and growth compared to untreated controls using the MTT assay. Four treatment schedules were tested: 72h concurrent, 72h inhibitor followed by 72h chemotherapeutic, 72h chemotherapeutic followed by 72h inhibitor, and a 144h concurrent dose with a media change at 72h (similar to the sequential treatments). CalcuSyn (BioSoft) was used to determine the combination index, which is a measurement of the type of drug interactions. A combination index (CI) of one indicates an additive response, less than one indicates a

synergistic response (greater than additive), and greater than one indicates an antagonistic response (less than additive).

Collection of mRNA for Cell Line Experiments. For each treatment, the SUM102 cells were grown in 15-cm dishes until 50-60% confluence. SUM102 cells were treated for 48h with a dose equivalent to two times the 72h-IC50 dose of each inhibitor (treated samples). To identify EGFR, MEK, and PI3K activation signatures, medium was removed after 48h of inhibitor treatment and replaced with fresh medium without inhibitor. mRNA was harvested at 4h, 8h, and 24h (post treatment samples). Cells were harvested by scraping, quickly placed into RNA lysis buffer, and mRNA was isolated using the Micro-FastTrack kit (Invitrogen).

Collection of RNA for Human Tumor Samples. 248 breast tissue samples represented by 241 fresh frozen breast tumor samples and 7 normal breast tissue samples were obtained from four different sources using IRB approved protocols from each participating institution: the University of North Carolina at Chapel Hill, The University of Utah, Thomas Jefferson University and the University of Chicago; many of these samples have appeared in previous publications [27-30], and 117 are new to this study (Supplementary Table 1). The patients were heterogeneously treated in accordance with the standard of care dictated by their disease stage, ER, and HER2 status.

Tumor sequence analysis. Tumor genomic DNA samples were isolated from 96 tumors using Qiagen DNeasy Kits according to the manufacturers protocol. Gene resequencing analyses were performed at Polymorphic DNA Technologies (Alameda, CA) using an ABI 3730xl DNA

sequencer and cycle sequencing, according to the manufacturers protocol. A two-step "boost/nested" PCR strategy was used where first a PCR reaction is performed to generate a larger DNA fragment, which is then used as a template for the nested reaction with a second set of PCR primers. Double stranded sequencing was performed on the nested product using the nested PCR primers as the sequencing primers. Exons 19 and 21 of *EGFR* were sequenced across all 96 patients, while exons 1 and 2 of *KRAS2*, 1 and 2 of *HRAS*, and 11 and 15 of *BRAF* were sequenced across 54 patients. No somatic alterations were detected.

Microarray Experiments. For the human tumor samples, the total RNA isolation and microarray protocols were performed as described in Hu *et al.* [5]; in this study, a number of tumor samples from previous studies were retested using a new custom Agilent microarray enriched for breast cancer genes. For cell lines experiments, labeled cRNA was generated from the mRNA using Agilent's Low RNA Input Linear Amplification Kit as described in Hu *et al.* [5]. For the cell line studies, the 48h inhibitor treated samples were compared to an untreated cell line reference to look for effects of an inhibitor, and for the post treatment samples, to identify an activation signature for that drug/pathway. Labeled experimental sample (Cy5 CTP) and reference (Cy3 CTP) were mixed and co-hybridized overnight on the same Custom 22K Agilent Human Whole Genome Oligonucleotide Microarray described above. Two to four microarrays per experimental cell line condition were performed, including a dye-flip replicate for gefitinib- and cetuximab-treated samples. Microarrays were scanned on an Axon GenePix 4000B microarray scanner and analyzed using GenePix Pro 5.1 software. Microarray raw data were uploaded into the UNC Microarray Database and Lowess normalization was performed on the Cy3 and Cy5 channels. The microarray and patient clinical data are available at UNC Microarray

Database [31] and have been deposited in the Gene Expression Omnibus under the accession number GSE6128.

Statistical Analyses. Intraclass correlations between cell line microarray experiments were performed to judge agreement between experiments as described in Hu *et al.* [5]. Unsupervised analyses of the cell line samples were performed by selecting genes with an absolute signal intensity of at least 30 units in both channels in at least 70% of the samples tested and that also showed a Log_2 R/G Lowess normalized ratio of two on at least two arrays. The program Cluster was used to hierarchically cluster samples and genes, and Treeview was used to view the data [32, 33]. Using the SUM102 treated cells, a one-class Significance Analysis of Microarrays (SAM) was used to identify significantly induced genes in all the post treatment experiments (two to three arrays for each experimental time point) [34]. Gene ontology enrichment was assessed using EASE [35].

Analyses of the primary tumor data used the top 500 induced genes from the cell line SAM analysis described above, after filtering for 30 units in both channels in at least 70% of the tumor samples. These genes were examined in a two-way hierarchical clustering analysis with the 248 UNC tumor sample set. Three distinct expression patterns were observed and labeled as Clusters #1-3. Next, the genes in each of these three tumor-defined clusters were identified in the NKI295 patient data set [36, 37], and a mean expression value for each cluster for each patient was determined. The NKI295 patients were then rank-ordered and separated into (a) two equal groups representing low and high, or (b) three equal groups representing low, medium, and high average expression for each cluster. In addition, similar gene-based rank order patient

stratifications were performed for individual genes that included *EGFR*, *HER2*, *HER4*, *EGF*, *TGFA*, *AREG*, *CRYAB, KRAS, KRAS*-amplicon profile, *HRAS, NRAS, PIK3CA, PIK3R1, AKT1, AKT2, AKT3, MEK1*, *MEK2, ERK1,* and *ERK2*. Survival analyses were performed using Cox-Mantel log-rank test in Winstat for Excel (R. Fitch Software). Multivariate Cox proportional hazards analysis was performed in SAS v9.0 (SAS Statistical Software, Cary, NC) to estimate the hazard ratio associated with cluster expression in the three groups after controlling for standard clinical predictors (age, ER status, size, grade, and node status). Chi Square tests (SAS v9.0) was used to examine correlations between cluster groups, individual genes, and tumor subtype.

Gene expression relative levels were visualized in relation to the EGFR signaling pathway using Cytoscape [38, 39]. The pathway was built *de novo* based on information from KEGG [40, 41], BioCarta [42], and a review by Yarden and Silowkoski [1] with a focus on the RAS-MEK and PI3K/AKT components. Using the 248 UNC breast tumor microarray dataset, an average gene expression profile is displayed for the Luminal A, Luminal B, basal-like, and HER2+/ER- tumors. Tumor "intrinsic" subtype was determined for each sample using the 306 gene Centroid Predictor described in Hu *et al.* [27]; the subtype classifications used for the NKI295 sample set were also derived from this same centroid predictor and are described in Fan *et al.* [43].

RESULTS

Cell line models of breast cancer. Breast cancer is a heterogeneous disease arising from at least two distinct epithelial cell populations; therefore, we selected cell lines models of basal-like and luminal cells to begin our investigations of the EGFR-pathway. The MCF-7 and ZR-75-1

cell lines were derived from breast tumors of luminal origin and have expression of CK8/18 and ER. Our previous studies examining cell lines of basal-like origin used immortalized human mammary epithelial cell lines (HMECs) [24, 25]; however, these lines are derived from normal rather than tumor tissue. Two ER-negative and HER2-non-amplified tumor-derived cell lines, SUM149 and SUM102, have been previously shown to express EGFR [18, 44] and show basal-like expression profiles [45]. The SUM102 and SUM149 lines share many characteristics with the basal-like tumors including expression of CK5/6, therefore, we used these two tumor-derived lines as *in vitro* models of basal-like breast cancers. By microarray analysis, EGFR gene expression was very low in the luminal cell lines and higher in the basal-like lines. EGFR protein expression by Western blot analysis was detectable in the basal-like lines, but not in the luminal lines (data not shown).

Drug sensitivity assays. To assess EGFR inhibitor sensitivity, the six cell lines described above were treated for 72h with a range of doses of gefitinib or cetuximab and an MTT assay was used to determine IC50 doses (Table 1). In response to gefitinib, the basal-like tumor-derived cell lines (SUM149 and SUM102) were two- to 100-fold more sensitive than the luminal lines. The two immortalized HMEC lines were also 33- and 50-fold more sensitive to gefitinib than the luminal lines, suggesting that the basal-like cell type as a whole is more sensitive to gefitinib versus the luminal cell type. Cetuximab sensitivity was observed in only a single cell line (SUM102, IC50=2ug/ml), with IC50 doses for MCF-7, ZR-75-1, SUM149, ME16C2, and HME-CC not achievable even with cetuximab doses as high as 100ug/ml. These cell lines were also treated with inhibitors that affect targets downstream of EGFR in its pathway including U1026 (MEK1/2 inhibitor) and LY294002 (PI3K inhibitor). Most of the cell lines had a similar level of

sensitivity to U0126 with the exception that SUM102 was approximately 5-fold more sensitive. IC50 doses for LY294002 were similar for most lines with the exception of ME16C and SUM149 cells, which were approximately 5-fold more resistant than the other lines. The SUM102 line was the only cell line that was sensitive to all four inhibitors and has previously been shown to be EGFR-dependent [44], and thus was chosen for further analyses of the EGFR-pathway.

Drug Combination Analyses. A phase II clinical trial is currently recruiting breast cancer patients who are ER-negative, PR-negative, and HER2 non-amplified (i.e. basal-like patients) to assess treatment responses to cetuximab alone or in combination with carboplatin [46]. A second phase II trial in an unselected population of metastatic breast cancer patients is also evaluating cetuximab in combination with carboplatin and irinotecan [47]. Therefore, we examined the combined effects of cetuximab and carboplatin, as well as three additional chemotherapeutics (doxorubicin, 5-fluorouracil, and paclitaxel), in SUM102 cells. We also tested the combined effects of gefitinib, U0126, and LY294002 with chemotherapeutic agents. Individual drug sensitivity (IC50 doses) for each chemotherapeutic was determined for all six cell lines (Table 2). The relative sensitivities varied across the cell lines and did not appear to correlate with cell type, with the exception that the two basal-like tumor-derived cell lines (SUM102 and SUM149) that were at least three-fold more sensitive to carboplatin, and at least two-fold more resistant to 5-fluorouracil when compared to their "normal" HMEC counterparts or the luminal cell lines.

The interaction of cetuximab with a chemotherapeutic in combination was examined solely in the SUM102 line because this was the only cetuximab sensitive line. As a starting point, we

treated SUM102 cells for 72h with cetuximab and a chemotherapeutic simultaneously. Synergistic interactions were not evident in any combination; all combinations were antagonistic as assessed by the method of Chou and Talalay in CalcuSyn [26] (Figure 1). We next analyzed the effect of sequential treatment: cells were treated for (a) 72h with cetuximab followed by 72h with chemotherapy, (b) 72h with chemotherapy followed by 72h with cetuximab, or (c) with cetuximab and chemotherapy simultaneously for 144h. Chemotherapy followed by cetuximab was generally more growth inhibitory than cetuximab followed by chemotherapy (Figure 1). The one exception was cetuximab with paclitaxel, where all sequence combinations were antagonistic (Figure 1). However, this antagonism may result from the high sensitivity to paclitaxel already observed in the SUM102 line. Carboplatin followed by cetuximab and the 144h concurrent treatments were synergistic even at low doses of both drugs. 5-fluorouracil followed a similar trend to that of carboplatin, while in the doxorubicin combinations synergy was only evident at doses higher than the IC50 dose for doxorubicin first or the 144h concurrent (Figure 1). Similar results were observed for combinations with gefitinib and LY294002 (a PI3K inhibitor) where chemotherapy followed by each inhibitor, and the 144h concurrent treatments, were more effective than the inhibitor first (data not shown). U0126 (a MEK inhibitor) combinations were different with chemotherapy first followed by U0126 being slightly less synergistic than the U0126 first or concurrent treatment; however, for U0126, all combinations except doxorubicin first, or paclitaxel first, were synergistic (data not shown).

EGFR-pathway gene expression patterns. To identify EGFR-dependent transcriptional patterns, we analyzed the gene expression data of the SUM102 cell line treated with EGFR inhibitors and then released from this inhibition. Using an unsupervised analysis, we

hierarchically clustered all time points from the cetuximab and gefitinib treatment experiments and identified over 500 genes that changed in expression at least 4-fold (Figure 2). Even though the two EGFR inhibitors have different mechanisms of inhibition, SUM102 cells treated for 48h with gefitinib or cetuximab showed very similar gene expression changes. Intraclass correlation (ICC) values between the gefitinib and cetuximab treated samples ranged from 0.627 to 0.934, and this level of similarity was also evident in the short dendrogram branches from the cluster analysis (Figure 2B). The post treatment samples (i.e. the inhibitor has been removed) that represent the reactivation of the EGFR-pathway were even more similar (ICC within each time point ranged from 0.862 to 0.962). A two-class SAM analysis to look for differences between gefitinib-post treatment samples versus cetuximab-post treatment samples identified only 58 significantly different genes with a false discovery rate (FDR) of 5%; thus, from a transcription standpoint, gefitinib and cetuximab elicited very similar results.

In response to gefitinib and cetuximab, the SUM102 cell line exhibited decreased expression of many proliferation genes (Figure 2). There was also a large cluster of transcripts that were induced by the inhibitors, consisting predominately of hypothetical genes with unknown functions. We were more interested in the genes induced after the removal of the inhibitor as this reflects the gene expression patterns associated with *de novo* activation of the EGFR-pathway. As early as 4h and 8h after inhibitor removal there was a substantial increase in expression for two ligands in the EGFR pathway, *amphiregulin* and *epiregulin*. *Cyclin A1* was also substantially increased (Figure 2C and D). Starting at 4h and continuing through 8h and 24h, genes with known roles in G1/S phase such as *CDC6*, *CDC*7, *TIMELESS*, and *ORCL6* were increased (Figure 2E and Supplemental Figure 1). By 8h and 24h, DNA synthesis and DNA

damage checkpoint genes were induced (Figure 2F). Classical gene expression-defined proliferation genes including *STK6* and *Cyclin B1* were highly induced by 24h (Figure 2G). There was also a repression of negative regulators of growth such as *Growth arrest-specific 1* and *Cyclin G2* (Supplemental Figure 1).

To objectively identify an EGFR-activation signature from the SUM102 cells, a one-class SAM analysis was used to identify genes that were statistically induced in the post treatment samples. Adjusting the SAM delta value to obtain the largest gene list with less than 5% FDR resulted in a gene list that was extremely large (10,017 genes, 4.97% FDR), therefore, the top 500 induced genes were selected for further analysis (0.02% FDR). This gene list was used to cluster 248 UNC breast tumor samples representing all five breast tumor subtypes (Figure 3 and Supplemental Figure 2). The list of induced genes from the *in vitro* experiments were not homogenously expressed across the tumor samples, and therefore to study these multiple expression patterns in the tumors, we defined "clusters" as any gene set that contained a minimum of 20 genes and a Pearson node correlation greater than 0.55. Using this criteria, we identified three clusters: Cluster #1 was high in a mix of breast tumor samples that contained all five breast cancer subtypes: Luminal B, Luminal A, basal-like, HER2+/ER- and normal-like samples (Figure 3C, far right dendrogram branch, 35 genes); Cluster #2 identified a set of tumors that was highly enriched for basal-like tumors and contained 58% of all basal-like tumors, 48% of all HER2+/ER- tumors and 3 Luminal B tumors (Figure 3D, center dendrogram branch, 27 genes); Cluster #3 was highly enriched for Luminal A and B tumors, and was also highly expressed in most of the HER2+/ER- and basal-like tumors that were also high for Cluster #2 (Figure 3E, left dendrogram branch– Luminal A and B tumors, and center dendrogram branch –

HER2+/ER- and basal tumors, 139 genes). Thus each gene cluster represents a stereotyped EGFR-activation signature that is enriched in a different subset of tumors (full gene lists for each cluster are in Supplemental Table 2). Gene Ontology (GO) analysis using EASE was performed on each gene cluster but only Cluster #3 had any significant GO terms, which were RNA processing, metabolism, binding, splicing, and modification (EASE scores < 0.05). However, *Cyclin E1* was present within Cluster #2 and is a known prognostic marker for breast cancer patients [48]; *Cyclin E1* is also associated with basal-like breast cancers [49, 50], which was recapitulated here, and known to be regulated by EGFR-signaling [51].

To examine the biological importance of these three gene sets, we individually applied them to a true test set of breast tumors (i.e. the NKI295 sample set described in [36, 37]) to determine whether they predicted patient outcomes. We first determined a mean expression value of all genes within each cluster for each patient. The patients were next rank-ordered based upon their mean expression values and divided into either two groups, or three groups, based upon their rank-order mean expression values. Kaplan-Meier survival analyses for Relapse-Free Survival (RFS) and Overall Survival (OS) were performed and all three clusters were statistically significant predictors of outcomes where the high expression always predicted a poor outcome (Figure 4 – OS; data not shown for RFS). Using a Cox multivariate analysis, we tested each group with the standard clinical parameters and determined that the high expression (top third) of Cluster #2 compared to the lowest expression (bottom third) significantly predicted a worse outcome for both RFS and OS (HR 2.63, 95% CI 1.44-4.79, p=0.0016 and HR 3.46, 95% CI 1.58-7.59, p=0.0019, respectively) after controlling for age, ER status, size, grade, and node status. Chi-squared analyses were performed to identify relations between tumor subtypes and

Clusters #1-3. Consistent with observations from Figure 3, the basal-like, luminal B, and HER2+/ER- tumors were associated with the high expression of all three clusters while the luminal A and normal-like samples rarely showed high expression (Table 3, p=<0.0001); in particular, the basal-like tumors were almost all high for Cluster #2 (89% in top 1/3).

Role of MEK and PI3K in the EGFR-Profile. Activation of EGFR leads to the downstream activation of numerous signaling pathways including the MEK/ERK and PIK3/AKT pathways [1]. To examine the role of these effectors, we treated the SUM102 cell line with the MEK1/2 inhibitor U0126 and the PI3K inhibitor LY294002 alone and in combination. Microarray time course experiments using inhibitor treated cells followed by inhibitor removal were conducted for U0126 and LY294002 using the experimental protocol as were done for cetuximab and gefitinib. The observed gene expression profiles for the MEK and the PI3K experiments were similar in both gene identity and direction when compared to the EGFR-profile, but gene expression changes were typically reduced in magnitude. The MEK and PI3K signatures were very similar to each other at the 4h and 8h time points (average ICC = 0.83), but diverged at 24h (average ICC = 0.59). Gene expression signatures of LY294002 and U0126 samples were also correlated with gefitinib and cetuximab gene expression signatures at 4h and 8h post treatment (LY294002 compared to gefitinib/cetuximab ICC = 0.83, U0126 compared to gefitinib/cetuximab ICC = 0.77). The LY294002 and U0126 24h post treatment samples were less correlated with gefitinib and cetuximab 24h post samples (LY294002 compared to gefitinib/cetuximab ICC = 0.51, U0126 Compared to gefitinib/cetuximab ICC = 0.41). We also treated cells with LY294002 and U0126 together to determine if the combined treatment would more completely recapitulate the EGFR activation profile; the 24h post combined treatment

samples showed a higher correlation value to the gefitinib and cetuximab samples (average ICC = 0.73), but still did not account for the entire gene expression pattern of the 24h post cetuximab and gefitinib treatments. These results suggest that the EGFR-profile could not be simply attributed to either the MEK or PIK3 pathway, but the combination of these two pathways with other downstream signaling pathways such as STATs [44], were more representative of the EGFR-signature than either pathway alone.

Potential mechanisms for activation of EGFR signaling *in vivo*. Activation of the EGFR-RAS-MEK pathway is known to occur via both ligand dependent and independent mechanisms. The empirically derived signatures of Clusters #1-3 are likely to include both. Thus, to distinguish between ligand dependent and independent mechanisms, we tested the gene expression patterns of the HER family of receptors (*EGFR, HER2, HER4*), some of their ligands (*TGFA, EGF, AREG*), as well as other pathway components including *MEK1, MEK2, PIK3CA, PIK3R1, CRYAB, AKT1-3*, the *RAS* proteins (H, K and N), *ERK1*, *ERK2*, and the *KRAS*-amplicon signature (identified and defined by gene expression in Herschkowitz et al. [52]) for their ability to predict patient outcomes, for correlations with tumor subtype (Table 3), and for correlations with Clusters #1-3 (Table 4). In this case, the potential 'ligand independent' gene list was not empirically defined, but was based on the existing literature for genes implicated as being able to activate the EGFR signaling pathway. Gene expression for individual genes was rank-ordered and divided into thirds as was done for Clusters #1-3 above. Each gene was tested for its ability to predict outcomes in the UNC 248 tumor data set. No individual gene listed above significantly predicted RFS and OS in both the UNC and NKI data sets. Associations between genes, or with Clusters #1-3, or with subtype were determined by Chi-square analysis

and identified many significant associations (Table 3). For example, high *HER2* expression, as expected, was significantly correlated with the HER2+/ER- subtype and *ER* expression was associated with both luminal subtypes (data not shown). *EGFR* expression was correlated with the basal-like subtype while high *HER4* and *PIK3R1* expression was associated with the luminal A subtype. Many other associations with the basal-like subtype were also evident that included the high expression of Clusters #1-3, *TGFA, AKT3*, *CRYAB, MEK1, NRAS* and the *KRAS*-amplicon signature and *KRAS* gene (Table 3). Other potentially biologically relevant associations included the high expression of Clusters #2 and #3, *HRAS, MEK1*, and *AKT1* with the HER2+/ER- subtype, and high expression of Clusters #1-3 and *HRAS* with the luminal B subtype. Thus, even though the Clusters #1-3 were identified using a basal-like tumor derived cell line, associations with luminal tumors were identified.

We also tested for associations between the high expression of Clusters #1-3 with the high expression (i.e. top 1/3 group) of each of the above-mentioned genes in both the UNC and NKI datasets (Table 4). In both datasets, the high expression of *MEK2* and *HRAS* was associated with Cluster 1, while the high expression of many other genes correlated with Clusters 2 and 3; of note was the high expression of the *KRAS*-amplicon, *HRAS, NRAS,* and *MEK1* with both Clusters #2 and #3, and the high expression of *EGFR* with only Cluster #2. The association of different genes with the three EGFR-activation signatures is likely reflective of the complexity of signaling in this pathway in breast cancer.

Lastly, an obvious mechanism for activation of the EGFR-RAS-MEK pathway is the somatic mutation of a *RAS* gene, *BRAF*, or *EGFR* itself, which are relatively frequent events in non-small

cell lung carcinomas. We performed resequencing analyses on a subset of the breast tumors analyzed by microarray for *EGFR* mutations in exons 19 and 20, which contain the ATP binding domain of EGFR, and for the common mutations in *HRAS, KRAS* and *BRAF*. No somatic sequence variants were detected in 96 tumors including 54 tumors that were over sampled for basal-like and HER2+/ER- tumors respectively.

Discussion

The epidermal growth factor receptor family is of tremendous biological and clinical importance for many solid epithelial tumors. In breast cancer patients, the response rate to single agent EGFR inhibitors has been low, however, these trials were performed on unselected patient populations [53, 54]. The EGFR-pathway has recently become a potential target in the basal-like subtype because at least 50% of basal-like tumors express EGFR as assessed by IHC [6]. These results lead to the initiation of a clinical trial for ER-negative, PR-negative, and HER2-nonamplified "triple-negative" (i.e. basal-like) breast cancers where patients will receive cetuximab alone versus cetuximab plus carboplatin. Our *in vitro* analyses show that all four basal-like cell lines were more sensitive to EGFR inhibitors compared to luminal cell lines. Only a single cell line (SUM102) was sensitive to cetuximab when EGF was present within the media, which is the condition that best mimics the *in vivo* environment [55]. We evaluated the combination of cetuximab and various chemotherapeutics in SUM102 cells and observed that the combination of cetuximab and carboplatin was highly synergistic at low doses of each drug. Even though the short-term co-treatment of cetuximab and carboplatin was antagonistic, synergism was observed in the long-term co-treatment suggesting that a combination of a EGFR inhibitor and chemotherapeutic might be a good combination against basal-like tumors.

Carboplatin, as well as other platinum derivatives, may also be good chemotherapeutic agents for basal-like breast cancers due to the implicated function of the BRCA1-pathway in this subtype. *BRCA1* mutation carriers are predisposed to develop tumors of the basal-like subtype [3, 56, 57]. In our basal-like tumor-derived cell lines, it has been reported that the SUM149 line has a *BRCA1* mutation and SUM102 line has barely detectable transcript levels of *BRCA1* [58]. From a mechanistic standpoint, *BRCA1* is required for repair of cisplatin induced DNA damage by recruiting *RAD51* to the site of damage [59, 60] and *BRCA1*-deficient cells exhibit increased sensitivity to cisplatin compared to wild type cells [61-64]. The combination of an EGFR inhibitor and a platinum drug has also been found to be synergistic in several other cell types [14, 65, 66]. In our experiments, we showed that not only are the basal-like tumor derived cell lines the most sensitive to carboplatin and the EGFR inhibitors when applied individually, but also that the combination was synergistic.

Given the importance of the EGFR pathway, we identified an EGFR-activation profile and examined its interplay with other biological features. *In vivo*, three distinct expression patterns were identified (Figure 3), of which two predicted patient outcomes in both the training and test data sets (i.e. Cluster #2 and #3). The greatest value of these activation signatures may be as an assay to identify tumors/patients that may benefit from therapeutic interventions in the EGFR-RAS-MEK pathway. These signatures may also represent a more dynamic descriptor of pathway activity compared to EGFR protein status alone, which does not predict responsiveness to EGFR inhibitors [67-69]. Microarray studies of breast cancer patients treated with EGFR inhibitors will be needed to address this hypothesis directly.

A critical question is what are the molecular events that cause activation of the EGFR-RAS-MEK pathway, and do these activation events vary with subtype? To address this question, we queried our data and found that potential activating events correlated with tumor subtype and with the EGFR-pathway activation profiles. These data are summarized in Figure 5 where many relationships were identified including previously known associations as well as new associations. For the Luminal A subtype, these data suggest that EGFR-RAS-MEK signaling is not a relevant therapeutic target and that if anything, the growth inhibitory *HER4* receptor may be the important HER family member for this subtype (Figure 5A); high expression of *HER4* and average expression of two of its ligands (*HB-EGF* and *NRG1*) were observed, and this subtype was also low for all three EGFR-activation signatures.

The Luminal B tumors showed moderate to high expression of the EGFR-activation signatures, high *HRAS* expression, and potentially high *MEK2* (Figure 5B). The EGFR/HER2 pathway has often been implicated as being at least partially responsible for tamoxifen resistance in ER+ patients [53, 70-74]. Our EGFR activation clusters were able to predict outcome differences in ER+ and tamoxifen-treated patients in both the UNC and NKI data sets (data not shown); but the expression of these clusters in ER+ patients closely parallels the genomic distinction of Luminal A versus Luminal B. These results suggest that part of the Luminal A versus Luminal B distinction is due to the activation of the EGFR/HER2 pathway in Luminal B tumors. Our results are also consistent with the hypothesis of the "non-genomic" effects of ER to activate the HER pathway, where membrane bound ER complexes with EGFR and/or HER2 to cause activation of the RAS-MEK and p38 pathways [70, 71, 75]. Studies have looked at gefitinib in combination

with an aromatase inhibitor in ER+/PgR+ tumors and found no additional benefit [74]. However, there may also be other downstream activations occurring such as high *HRAS* expression. Methods to effectively target this pathway in Luminal B tumors are not clear, but our data suggests that agents that target *RAS* or *MEK* may be worth investigating.

The HER2+/ER- tumors, as expected, showed high expression of *HER2* and were also associated with high *HRAS* and *MEK1/MEK2* (Figure 5C). High *AKT1* levels were also associated with this tumor subtype, which has been previously identified [76, 77]. Targeting of the HER pathway in the HER2+/ER- tumors involves the administration of trastuzumab; however, given that the response rate to trastuzumab-containing therapies is approximately 50%, additional agents are needed. Candidates from this study could include the direct or indirect targeting of *AKT1* and/or *MEK*1.

The data for basal-like patients suggest that EGFR-RAS-MEK pathway activation is a requisite as almost every basal-like tumor analyzed showed the high expression of one or more of the EGFR-activation signatures. High expression of many of the genes in the pathway were significantly correlated with the basal-like subtype including *EGFR, TGFA, MEK1, MEK2, AKT3, CRYAB, NRAS* and the *KRAS*-amplicon signature (Figure 5D). The potential mechanisms of EGFR pathway activation appears to vary within the basal-like tumors and most lean towards ligand-independent mechanisms. One potential mechanism involves CRYAB; Moyano *et al*. showed that the ectopic expression of CRYAB in breast epithelial cells caused them to become transformed and EGF-independent through activation of the MEK/ERK pathway [21]. This transformed phenotype was reverted by the addition of the MEK inhibitors PD98059 and U0126,

while the PIK3 inhibitor LY294002 had little effect. CRYAB may also confer resistance to EGFR inhibitors as well as chemotherapy by its anti-apoptotic mechanism, which is the inhibition of caspase-3 activation [78, 79]. A second mechanism of activation is via high *RAS* expression; interestingly, the expression of the *KRAS*-amplicon gene expression signature showed stronger associations versus the *KRAS* gene alone, suggesting that either the amplicon signature is a better assay for detecting *KRAS* activity than the simple expression of *KRAS* alone, or more likely, that additional co-amplified and highly expressed genes synergize with *KRAS* to more potently activate the RAS-MEK pathway. Only a small subset of basal-like tumors showed the high expression of *EGFR* and one of its ligands (typically *TGFA*) and the low expression of *CRYAB* and *KRAS*. It is only this subset of basal-like tumors that might be responsive to EGFR inhibitors because it is only these tumors that might activate this pathway using a ligand-dependent mechanism. Examples of individual basal-like tumors that show each of these activation profiles is presented in Figure 6.

Conclusions

The effective targeting of the EGFR-RAS-MEK pathway in basal-like tumors may require additional patient stratification based upon the mechanism of activation of the pathway. For example, those basal-like patients who show high *EGFR* and ligand and low *CRYAB* and *KRAS*-amplicon could be candidates for EGFR inhibitors. While those who show high *CRYAB* and/or *KRAS*-amplicon could be candidates for MEK inhibitors, of which many are in clinical development. Alternatively, if MEK inhibitors show good efficacy and low side effects, all patients who show activation of the EGFR-RAS-MEK pathway as assessed by Clusters #2 and #3 (both of which were associated with high *MEK* expression-Table 4), may prove to be

candidates for these drugs, which would include all basal-like patients, as well as some HER2+/ER- and Luminal B patients.

Abbreviations

EGFR/HER1: epidermal growth factor receptor; ER: estrogen receptor; HER: human epidermal growth factor receptor; MTT: mitochondrial dye conversion assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; CI: Combination Index; SAM: Significance Analysis of Microarrays; HMEC: human mammary epithelial cell; FDR: false discovery rate; GO: gene ontology; RFS: relapse-free survival; OS: overall survival; HR: hazard ratio; 95% CI: 95% confidence interval; ICC: Intraclass correlation; PgR: progesterone receptor.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

K.A.H. performed the cell line experiments, cell line and tumor data analysis, drafted the paper and helped with the design of the study. V.J.W., C.F., M.A.T. assisted with data analysis. C.I.S. made initial observations of EGFR dependency of SUM102 cells and assisted with the discussion. Tumor sample collection, clinical data acquisition and interpretations was accomplished by L.A.C, L.R.S., T.R.H, P.S.B. X.H. performed tumor RNA preparation and microarray experiments for tumor samples. C.M.P was the Principal Investigator, instigated and designed the study, and helped draft the paper.

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Figure 1. Effects of different combination schedules of cetuximab with chemotherapeutics in SUM102 cells. Cells were treated with four different combination schedules: 1) 72h cetuximab followed by 72h chemotherapy, 2) 72h chemotherapy followed by 72h cetuximab, 3) 72h concurrent chemotherapy and cetuximab, and 4) 144h concurrent chemotherapy and cetuximab. **A)** Growth inhibitory effects of cetuximab and carboplatin combinations. **B)** Combination analysis of cetuximab and carboplatin treatments. **C)** Growth inhibitory effects of cetuximab and paclitaxel combinations. **D)** Combination analysis of cetuximab and paclitaxel treatments. **E)** Growth inhibitory effects of cetuximab and 5-fluorouracil combinations. **F)** Combination analysis of cetuximab and 5-fluorouracil treatments. **G)** Growth inhibitory effects of cetuximab and doxorubicin combinations. **H)** Combination analysis of cetuximab and doxorubicin treatments. Combination Index (CI) values below one are synergistic, equal to one are additive, and greater than one are antagonistic.

Figure 2. Gene expression patterns for SUM102 cells treated with gefitinib or cetuximab. Unsupervised hierarchical cluster analysis was performed on 48h inhibitor treated and 4h, 8h, and 24h post 48hr inhibitor treated samples. **A)** The complete cluster overview with the colored bars indicating the location of the clusters shown in C-G. **B)** Close up of the experimental sample associated dendrogram. **C+D)** 4h and 8h post treatment induced genes including the EGFR ligands *Amphiregulin* and *Epiregulin*. **E)** Genes involved with the G1/S phase transition induced beginning in the 4h post inhibitor and continuing though 24h. **F)** Genes involved in DNA synthesis induced at 8h post inhibitor and continuing through 24h. **G)** Proliferation genes typically observed in tumor derived profiles including *STK6* and *Cyclin B1*.

Figure 3. *In vivo* EGFR-activation profiles and additional genes implicated in the EGFR-RAS-MEK pathway analyzed using the UNC 248 tumor data set. **A)** The top 500 induced genes from the SUM102 post treatment experiments were hierarchical clustered using the 248 UNC tumors. Colored bars indicate the location of the three clusters in D-E. **B)** Tumor associated dendrogram color coded according to tumor subtype: Luminal A – dark blue, Luminal B – light blue, true normals and normal-like – green, HER2+/ER- - pink, and basal-like – red. **C)** Cluster #1 that identified a mixed group of tumors. **D)** Selected genes from the center of Cluster #2 that are high in most basal-like tumors. **E)** Selected genes from the center of Cluster #3 that are high in the luminal tumors. **F)** Data for genes with suggested roles in EGFR-pathway. **G)** Data for the *KRAS*-amplicon signature identified in Herschkowitz *et al.* [52].

Figure 4. Kaplan-Meier survival plots for the 295 NKI tumors/patients using expression from the three different *in vivo* defined EGFR-activation profiles. The average expression value for each cluster in each patient was determined and the patients then put into rank-order and divided into two equal groups or three equal groups. Overall survival analysis was performed for each cluster. X indicates censored data due loss to follow-up or to information at last checkup. Note that Clusters #2 and #3 were also similarly prognostic for the UNC 248 training data set.

Figure 5. EGFR pathway diagram displayed for each breast tumor subtype. The average gene expression value for each gene within each subtype is displayed for the EGFR-pathway and for the three EGFR-activation profiles using the UNC 248 tumor dataset. Eight genes from the middle of each of the three EGFR-activation clusters were used to view expression of the clusters in each of the subtypes. A pink node border identifies the genes that showed statistically

significant associations with subtype. *Note: the NKI HER4 data spot was used since HER4 was not present in the UNC data set. **A)** Luminal A, **B)** Luminal B, **C)** HER2+/ER- and **D)** Basal-like.

Figure 6. EGFR pathway diagram displayed for each type of mechanism that could cause activation of the EGFR-RAS-MEK pathway in basal-like tumors. A) *EGFR*-ligand dependent activation profile, B) *CRYAB* activation profile, C) *KRAS*-amplicon activation profile, D) multiple simultaneous activation profiles.

Supplemental Figure 1. Full cluster diagram for the gene expression patterns of SUM102 cells treated with gefitinib or cetuximab.

Supplemental Figure 2. Full cluster diagram for the *in vivo* EGFR-activation profiles clustered on the UNC tumor data set.

Supplemental Table 1. Clinical data associated with each tumor sample.

Supplemental Table 2. Genes from Cluster #1-3 identified from the 500 SUM102 genes clustered on the UNC tumor dataset.

Table 1. Estimated IC50 doses of six breast cell lines for the EGFR inhibitors gefitinib, cetuximab, the MEK1/2 inhibitor U0126, and the PI3K inhibitor LY294002

Cell Line	Gefitinib (μM)	Cetuximab (μg/mL)	U0126 (uM)	LY294002 (uM)
ME16C	0.3 (0.02)	>100[a]	19.7 (0.66)	21.2 (0.63)
HME-CC	0.2 (0.01)	>100[a]	12.7 (0.33)	7.3 (0.17)
SUM102	0.1 (0.002)	2.3 (0.15)	4.3 (0.20)	3.4 (0.10)
SUM149	4.7 (0.14)	>100[a]	21.8 (0.80)	18.4 (0.48)
MCF-7	21.1 (0.29)	>100[a]	17.0 (1.15)	3.9 (0.13)
ZR-75-1	11.1 (0.12)	>100[a]	25.0 (0.74)	2.4 (0.05)

Note that the standard errors are presented within ()

[a]No achievable IC50 dose with doses up to 100μg/mL

Table 2. Estimated IC50 doses of six breast cell lines treated with chemotherapeutics

Cell Line	5-Florouracil (uM)	Doxorubicin (nM)	Carboplatin (uM)	Paclitaxel (nM)
ME16C	6.0 (0.29)	32.8 (1.89)	37.5 (0.63)	0.052 (0.004)
HME-CC	1.1 (0.07)	35.5 (3.26)	48.3 (1.41)	0.025 (0.003)
SUM102	16.8 (0.82)	5.1 (0.27)	11.7 (0.26)	0.00057 (0.00001)
SUM149	28.6 (1.33)	45.0 (3.06)	7.7 (0.24)	0.71 (0.006)
MCF-7	1.2 (0.15)	56.9 (4.26)	89.4 (3.79)	0.23 (0.02)
ZR-75-1	8.4 (1.06)	26.5 (1.39)	62.6 (1.98)	0.99 (3.34)

Note that the standard errors are presented within ().

Table 3. Chi-square analysis for association of gene expression with subtypes. Samples were rank ordered into three equal groups and the percentage of each subtype in the highest expression group is reported for the NKI patient data set.

	Basal-like	HER2+/ER-	Luminal A	Luminal B	Normal-like	p-value
# tumors	53	35	123	55	29	
Cluster 1[a]	68%	37%	12%	56%	14%	<0.0001
Cluster 2[a]	89%	49%	5%	49%	7%	<0.0001
Cluster 3[a]	77%	51%	11%	47%	0%	<0.0001
EGFR[a]	68%	20%	27%	18%	41%	<0.0001
HER2[a]	15%	100%	28%	26%	24%	<0.0001
HER4*	9%	3%	50%	38%	31%	<0.0001
TGFA[b]	74%	37%	17%	25%	38%	<0.0001
AREG[a]	3%	34%	43%	35%	41%	<0.0001
EGF	17%	40%	37%	36%	31%	0.23
CRYAB[a]	70%	11%	33%	4%	48%	<0.0001
KRAS amplicon[a]	68%	40%	24%	35%	0%	<0.0001
KRAS gene[c]	32%	37%	33%	38%	21%	0.36
HRAS[d]	32%	66%	17%	64%	7%	<0.0001
NRAS[a]	70%	28%	17%	44%	21%	<0.0001
PIK3CA	30%	17%	36%	36%	41%	0.28
PIK3R1[a]	21%	14%	42%	25%	55%	0.0012
AKT1[a]	26%	63%	27%	40%	24%	<0.0001
AKT2*	26%	40%	27%	47%	38%	0.26
AKT3[a]	51%	14%	39%	9%	45%	<0.0001
MEK1	53%	46%	25%	29%	24%	0.023
MEK2[e]	42%	43%	25%	42%	24%	0.068
ERK1[f]	30%	26%	31%	42%	41%	0.49
ERK2[g]	40%	31%	26%	45%	31%	0.048

*Note: HER4 could not be assessed in UNC data due to too many missing values; HER3 was not present in the NKI data set; AKT2 was not present in the UNC data set

[a] associations were also similarly significant in the UNC sample set

[b] nominally significant in UNC data (p-value=0.0046)

[c] nominally significant association in the UNC data (p-value= 0.0051)

[d] nominally significant in the UNC data (p-value = 0.003)

[e] nominally significant in the UNC data (p-value = 0.0023)

[f] significant in the UNC data (p-value = 0.0003)

[g] significant in the UNC data (p-value = <0.0001)

Bonferroni corrected level of significance α=0.0022

Table 4. Associations between Clusters #1-3 and individual genes using the NKI295 sample set. Chi-squared analyses were used to identify associations between the high expression of the individual EGFR-activation profiles for each cluster (top 1/3) and the expression of individual genes categorized as high (top 1/3). The % of tumors with the high expression of each cluster and that show the high expression of the individual gene is shown.

	Cluster 1		Cluster 2		Cluster 3	
	%	p-val	%	p-val	%	p-val
EGFR	39%	0.1783	43%	0.0091[b]	38%	0.15
HER2	26%	0.0017	25%	<0.0001[c]	24%	<0.0001[a]
HER4*	21%	<0.0001	12%	<0.0001	18%	<0.0001
TGFA	40%	0.0665	48%	0.0002	47%	0.0021
AREG	22%	0.0007[c]	23%	<0.0001[a]	28%	0.064[f]
EGF	35%	0.1380	25%	0.0691	27%	0.033[d]
CRYAB	35%	0.3214[f]	38%	0.0524	38%	0.0013
KRAS amplicon	38%	0.1973[e]	52%	<0.0001[c]	63%	<0.0001[a]
KRAS gene	27%	0.0022[a]	31%	0.8795	36%	0.14[e]
HRAS	48%	<0.0001[c]	51%	<0.0001	47%	0.0018
NRAS	45%	0.0362	56%	<0.0001[c]	59%	<0.0001[a]
PIK3ca	22%	0.0032[b]	27%	0.1415[e]	30%	0.33[e]
PIK3R1	24%	0.0009[a]	20%	<0.0001[a]	19%	<0.0001
AKT1	41%	0.0112	39%	0.0899	34%	0.36
AKT2*	40%	0.0519	37%	0.3524	33%	0.94
AKT3	26%	0.0004	33%	0.1569	35%	0.64[f]
MEK1	39%	0.0335	47%	0.0032[d]	48%	<0.0001
MEK2	58%	<0.0001[a]	44%	0.0113[d]	36%	0.55[f]
ERK1	37%	0.0718[e]	23%	0.0009[c]	19%	<0.0001[a]
ERK2	39%	0.0238	37%	0.3457[e]	36%	0.46[e]

*Note: HER4 could not be assessed in UNC data due to too many missing values; HER3 was not present in the NKI data set; AKT2 was not present in the UNC dataset.

[a] the statistically significant association was also significant in the UNC data set ($p<0.0025$).

[b] the association was nominally significant in the NKI dataset ($p<0.05$), but significant in the UNC dataset ($p<0.0025$).

[c] the association was significant in the NKI dataset ($p<0.0025$), but nominally significant in the UNC dataset ($p<0.05$).

[d] the association was nominally significant in both datasets ($p<0.05$).

[e] the association was significant in UNC dataset ($p<0.0025$).

[f] the association was nominally significant in the UNC dataset ($p<0.05$).

Bonferroni corrected level of significance $\alpha=0.0025$

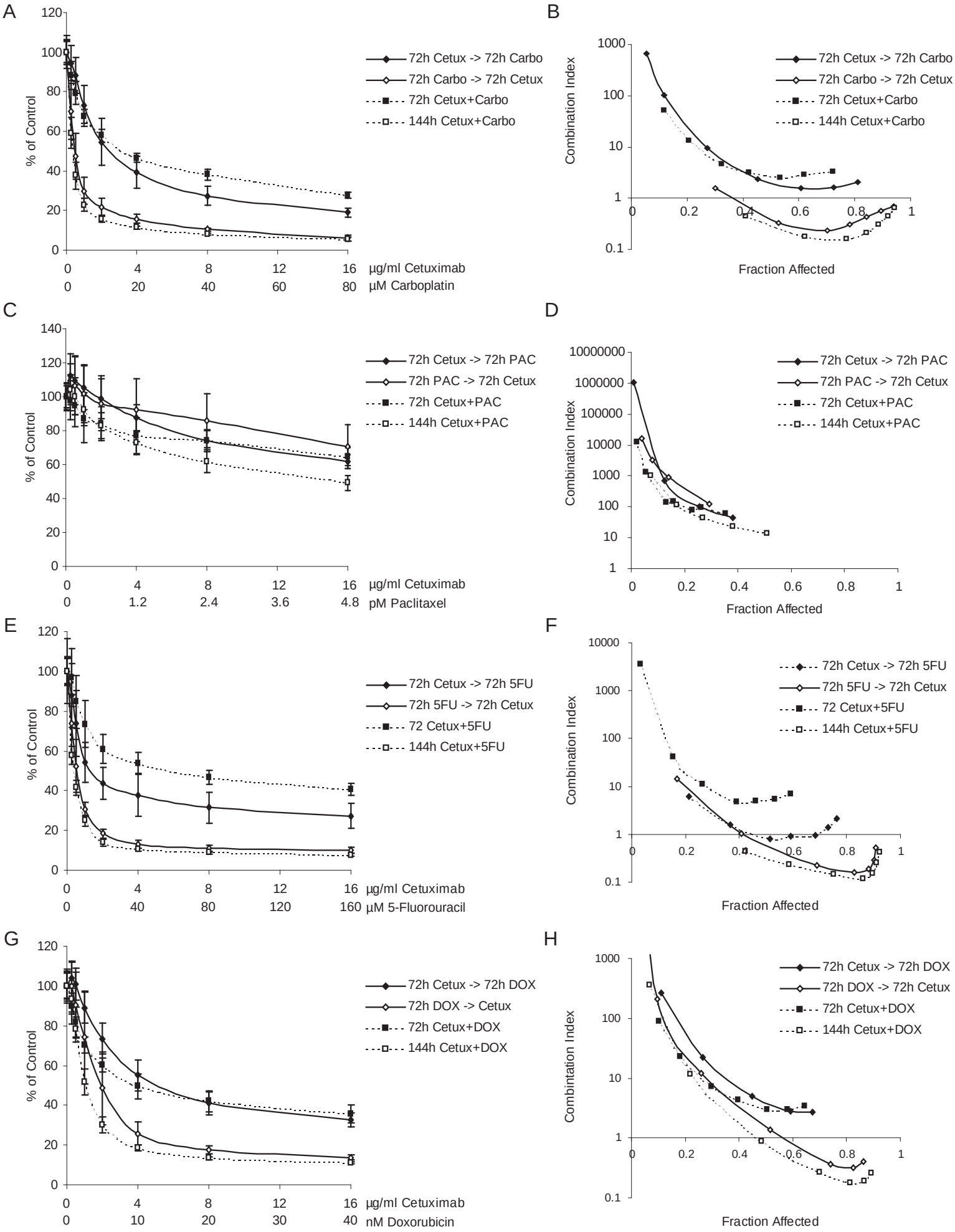
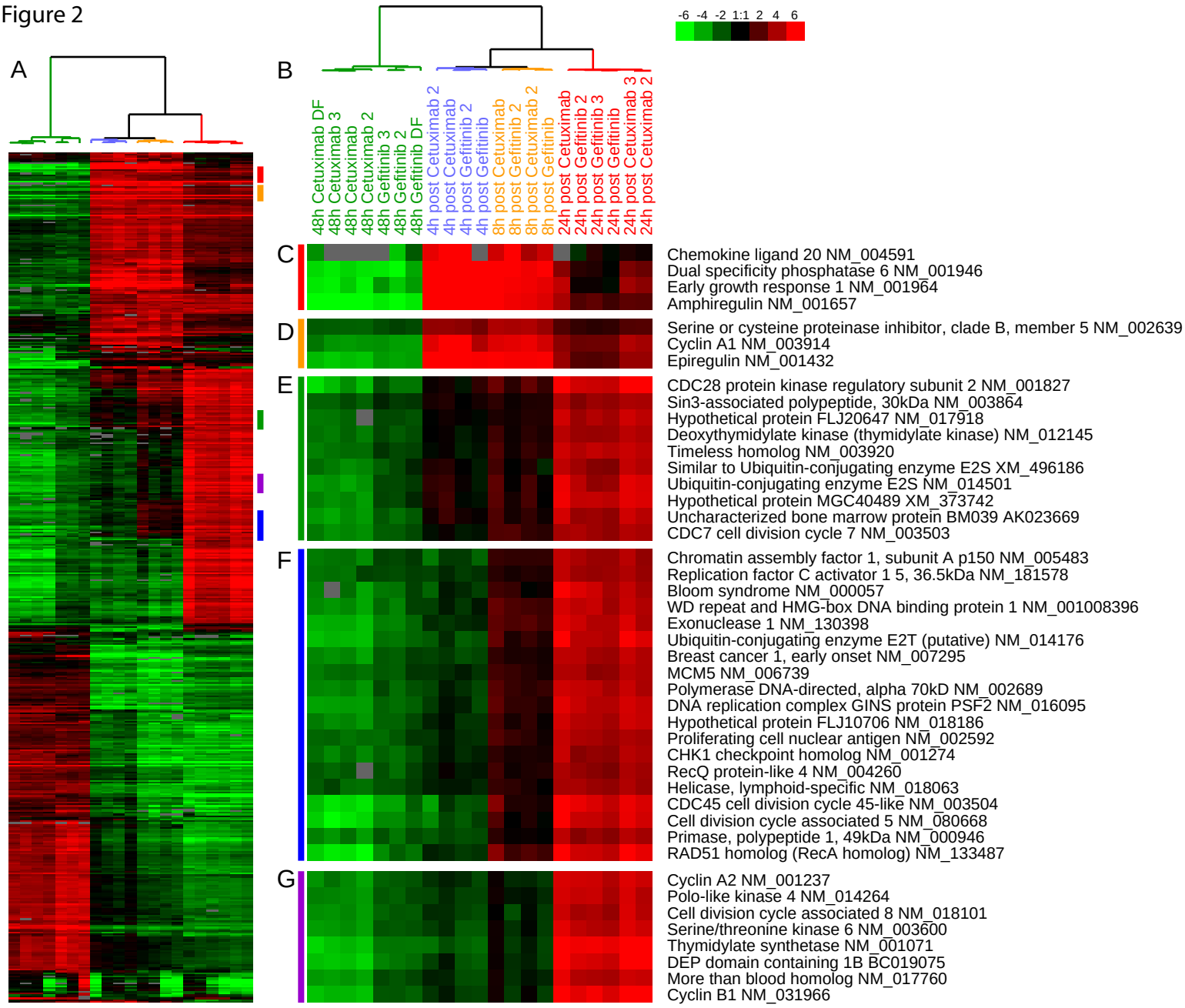

A
% of Control
72h Cetux -> 72h Carbo
72h Carbo -> 72h Cetux
72h Cetux+Carbo
144h Cetux+Carbo
µg/ml Cetuximab
µM Carboplatin
B
Combination Index
Fraction Affected
C
% of Control
72h Cetux -> 72h PAC
72h PAC -> 72h Cetux
72h Cetux+PAC
144h Cetux+PAC
µg/ml Cetuximab
pM Paclitaxel
D
Combination Index
Fraction Affected
E
% of Control
72h Cetux -> 72h 5FU
72h 5FU -> 72h Cetux
72 Cetux+5FU
144h Cetux+5FU
µg/ml Cetuximab
µM 5-Fluorouracil
F
Combination Index
Fraction Affected
G
% of Control
72h Cetux -> 72h DOX
72h DOX -> Cetux
72h Cetux+DOX
144h Cetux+DOX
µg/ml Cetuximab
nM Doxorubicin
H
Combintation Index
Fraction Affected

Figure 1

Figure 2



Chemokine ligand 20 NM_004591
Dual specificity phosphatase 6 NM_001946
Early growth response 1 NM_001964
Amphiregulin NM_001657

Serine or cysteine proteinase inhibitor, clade B, member 5 NM_002639
Cyclin A1 NM_003914
Epiregulin NM_001432

CDC28 protein kinase regulatory subunit 2 NM_001827
Sin3-associated polypeptide, 30kDa NM_003864
Hypothetical protein FLJ20647 NM_017918
Deoxythymidylate kinase (thymidylate kinase) NM_012145
Timeless homolog NM_003920
Similar to Ubiquitin-conjugating enzyme E2S XM_496186
Ubiquitin-conjugating enzyme E2S NM_014501
Hypothetical protein MGC40489 XM_373742
Uncharacterized bone marrow protein BM039 AK023669
CDC7 cell division cycle 7 NM_003503

Chromatin assembly factor 1, subunit A p150 NM_005483
Replication factor C activator 1 5, 36.5kDa NM_181578
Bloom syndrome NM_000057
WD repeat and HMG-box DNA binding protein 1 NM_001008396
Exonuclease 1 NM_130398
Ubiquitin-conjugating enzyme E2T (putative) NM_014176
Breast cancer 1, early onset NM_007295
MCM5 NM_006739
Polymerase DNA-directed, alpha 70kD NM_002689
DNA replication complex GINS protein PSF2 NM_016095
Hypothetical protein FLJ10706 NM_018186
Proliferating cell nuclear antigen NM_002592
CHK1 checkpoint homolog NM_001274
RecQ protein-like 4 NM_004260
Helicase, lymphoid-specific NM_018063
CDC45 cell division cycle 45-like NM_003504
Cell division cycle associated 5 NM_080668
Primase, polypeptide 1, 49kDa NM_000946
RAD51 homolog (RecA homolog) NM_133487

Cyclin A2 NM_001237
Polo-like kinase 4 NM_014264
Cell division cycle associated 8 NM_018101
Serine/threonine kinase 6 NM_003600
Thymidylate synthetase NM_001071
DEP domain containing 1B BC019075
More than blood homolog NM_017760
Cyclin B1 NM_031966

Figure 3

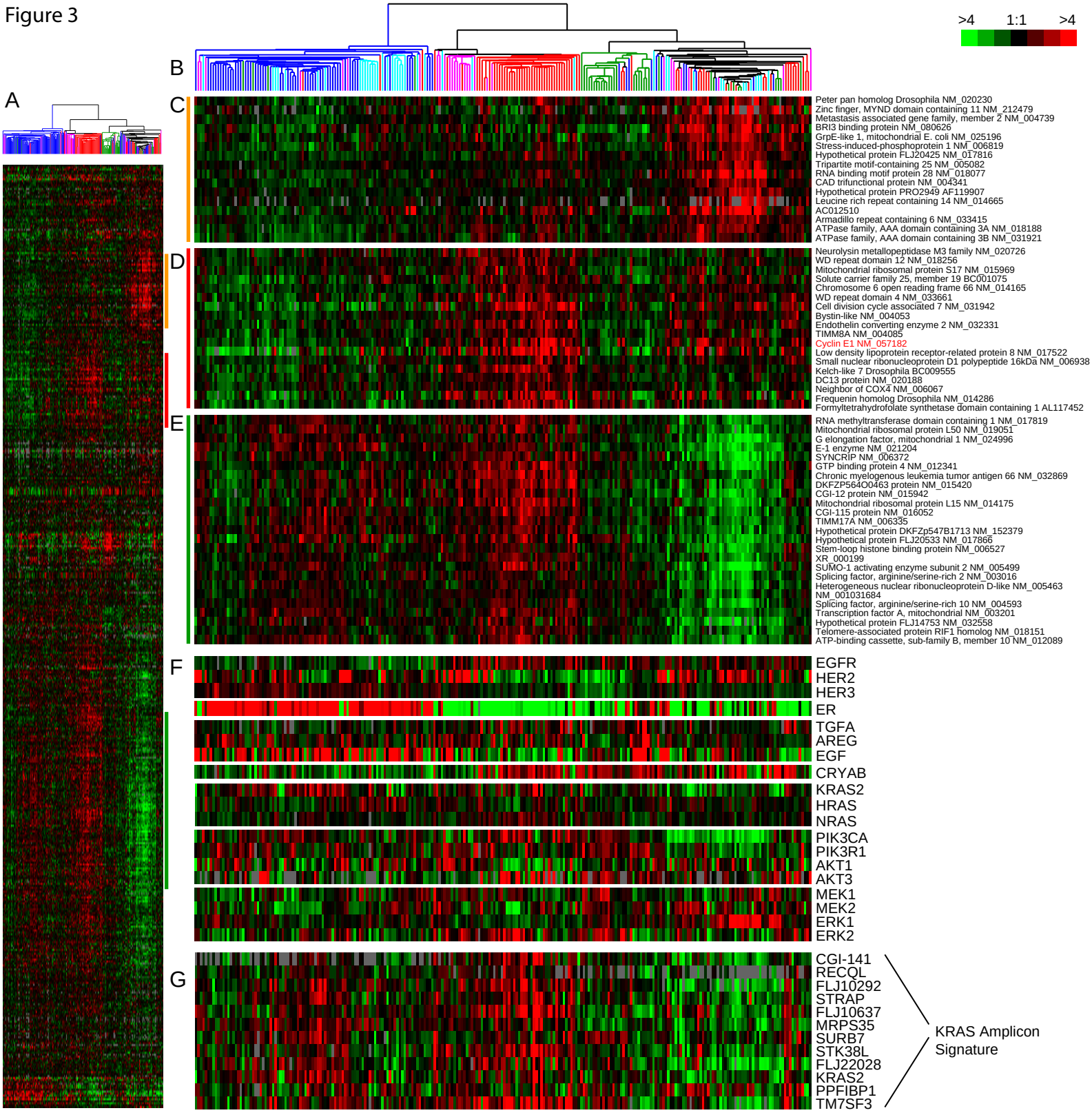

>4
1:1
>4
A
B
C
Peter pan homolog Drosophila NM_020230
Zinc finger, MYND domain containing 11 NM_212479
Metastasis associated gene family, member 2 NM_004739
BRI3 binding protein NM_080626
GrpE-like 1, mitochondrial E. coli NM_025196
Stress-induced-phosphoprotein 1 NM_006819
Hypothetical protein FLJ20425 NM_017816
Tripartite motif-containing 25 NM_005082
RNA binding motif protein 28 NM_018077
CAD trifunctional protein NM_004341
Hypothetical protein PRO2949 AF119907
Leucine rich repeat containing 14 NM_014665
AC012510
Armadillo repeat containing 6 NM_033415
ATPase family, AAA domain containing 3A NM_018188
ATPase family, AAA domain containing 3B NM_031921
D
Neurolysin metallopeptidase M3 family NM_020726
WD repeat domain 12 NM_018256
Mitochondrial ribosomal protein S17 NM_015969
Solute carrier family 25, member 19 BC001075
Chromosome 6 open reading frame 66 NM_014165
WD repeat domain 4 NM_033661
Cell division cycle associated 7 NM_031942
Bystin-like NM_004053
Endothelin converting enzyme 2 NM_032331
TIMM8A NM_004085
Cyclin E1 NM_057182
Low density lipoprotein receptor-related protein 8 NM_017522
Small nuclear ribonucleoprotein D1 polypeptide 16kDa NM_006938
Kelch-like 7 Drosophila BC009555
DC13 protein NM_020188
Neighbor of COX4 NM_006067
Frequenin homolog Drosophila NM_014286
Formyltetrahydrofolate synthetase domain containing 1 AL117452
E
RNA methyltransferase domain containing 1 NM_017819
Mitochondrial ribosomal protein L50 NM_019051
G elongation factor, mitochondrial 1 NM_024996
E-1 enzyme NM_021204
SYNCRIP NM_006372
GTP binding protein 4 NM_012341
Chronic myelogenous leukemia tumor antigen 66 NM_032869
DKFZP564O0463 protein NM_015420
CGI-12 protein NM_015942
Mitochondrial ribosomal protein L15 NM_014175
CGI-115 protein NM_016052
TIMM17A NM_006335
Hypothetical protein DKFZp547B1713 NM_152379
Hypothetical protein FLJ20533 NM_017866
Stem-loop histone binding protein NM_006527
XR_000199
SUMO-1 activating enzyme subunit 2 NM_005499
Splicing factor, arginine/serine-rich 2 NM_003016
Heterogeneous nuclear ribonucleoprotein D-like NM_005463
NM_001031684
Splicing factor, arginine/serine-rich 10 NM_004593
Transcription factor A, mitochondrial NM_003201
Hypothetical protein FLJ14753 NM_032558
Telomere-associated protein RIF1 homolog NM_018151
ATP-binding cassette, sub-family B, member 10 NM_012089
F
EGFR
HER2
HER3
ER
TGFA
AREG
EGF
CRYAB
KRAS2
HRAS
NRAS
PIK3CA
PIK3R1
AKT1
AKT3
MEK1
MEK2
ERK1
ERK2
G
CGI-141
RECQL
FLJ10292
STRAP
FLJ10637
MRPS35
SURB7
STK38L
FLJ22028
KRAS2
PPFIBP1
TM7SF3
KRAS Amplicon Signature

Figure 4

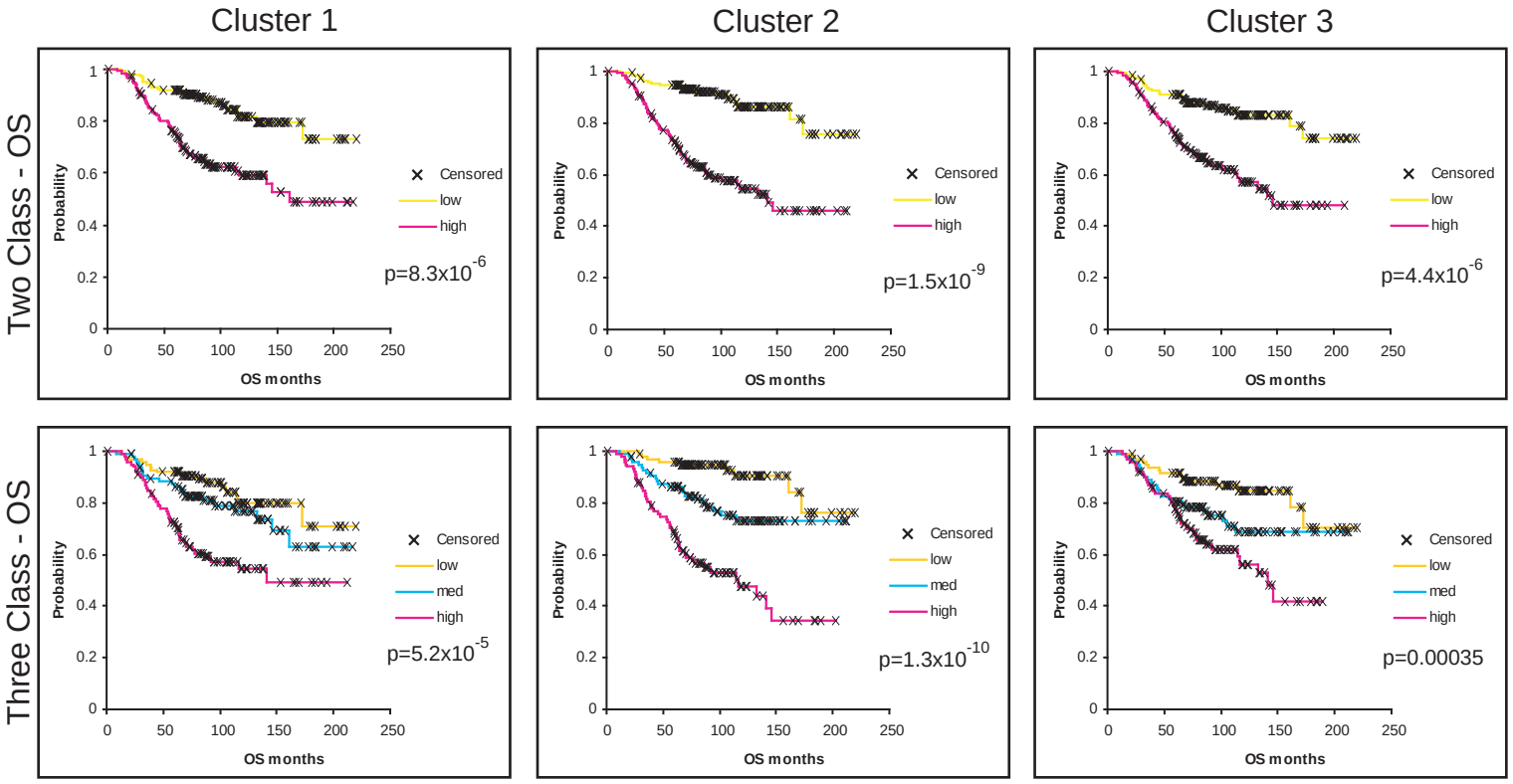

Cluster 1
Cluster 2
Cluster 3
Two Class - OS
Three Class - OS
Probability
OS months
Censored
low
med
high
p=8.3x10-6
p=1.5x10-9
p=4.4x10-6
p=5.2x10-5
p=1.3x10-10
p=0.00035

Figure 5

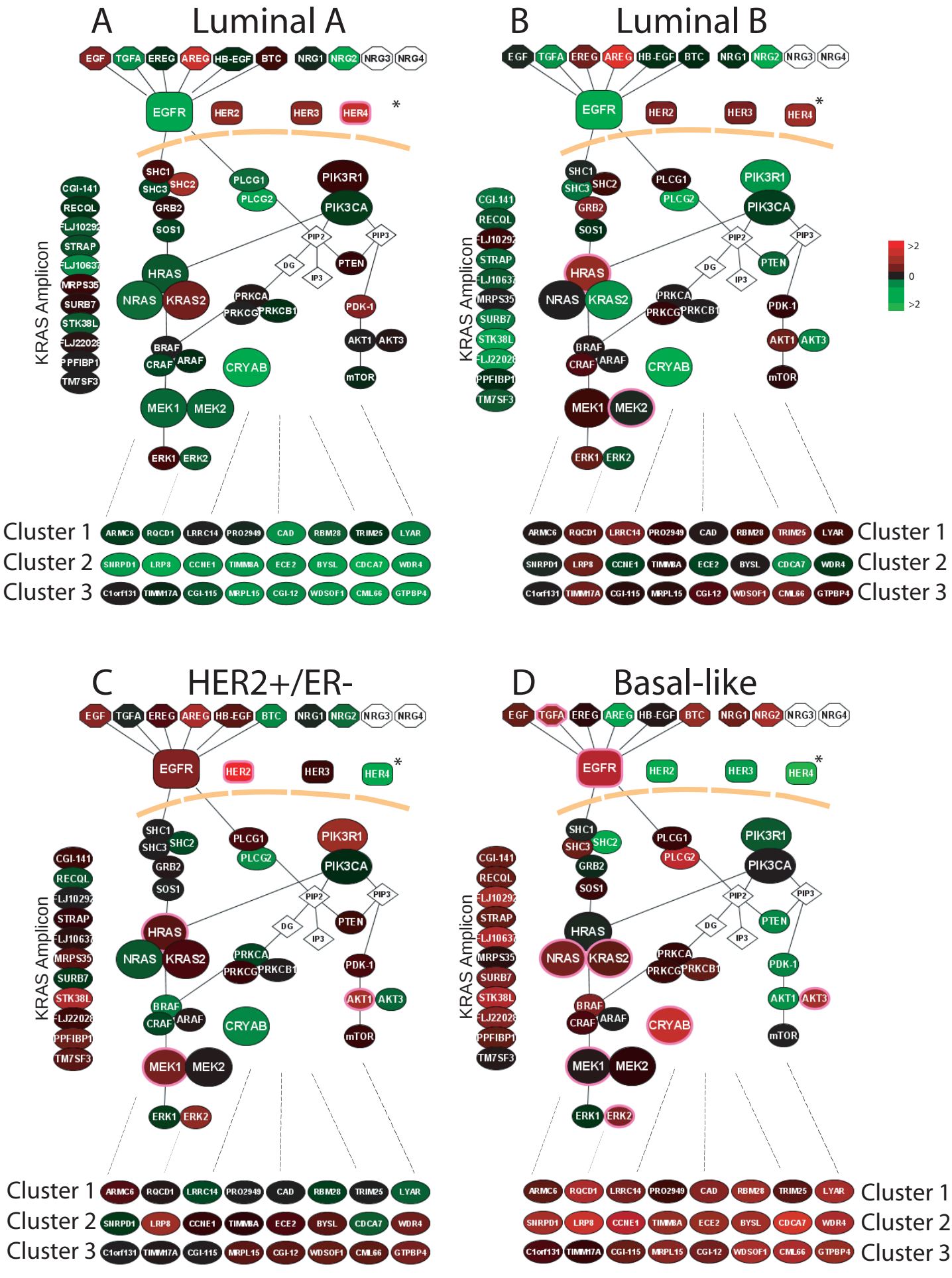
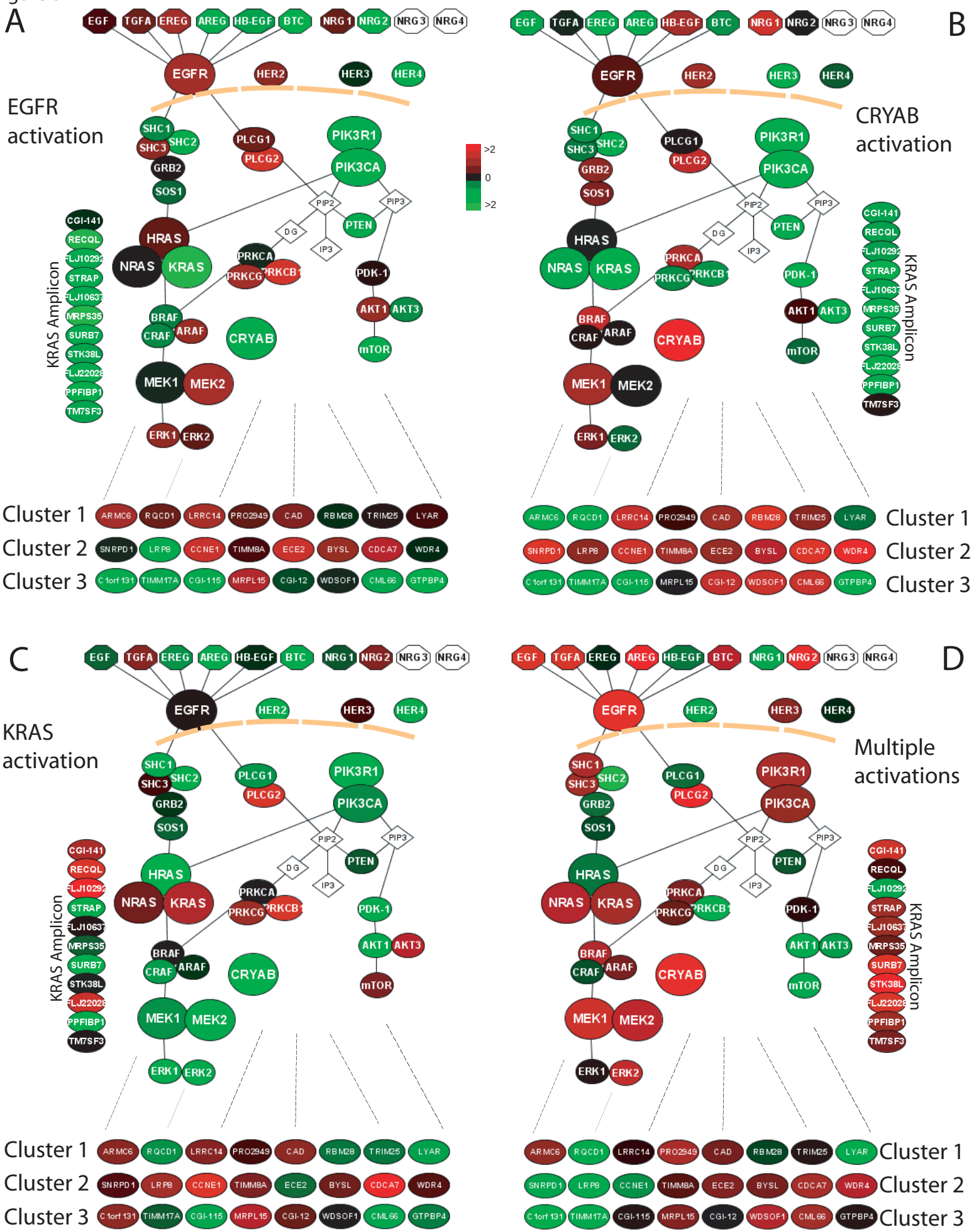

A
Luminal A
B
Luminal B
C
HER2+/ER-
D
Basal-like
EGF
TGFA
EREG
AREG
HB-EGF
BTC
NRG1
NRG2
NRG3
NRG4
EGFR
HER2
HER3
HER4
SHC1
SHC3
SHC2
GRB2
SOS1
PLCG1
PLCG2
PIK3R1
PIK3CA
PIP2
PIP3
DG
IP3
PTEN
HRAS
NRAS
KRAS2
PRKCA
PRKCG
PRKCB1
PDK-1
AKT1
AKT3
mTOR
BRAF
CRAF
ARAF
CRYAB
MEK1
MEK2
ERK1
ERK2
KRAS Amplicon
CGI-141
RECQL
FLJ10292
STRAP
FLJ10637
MRPS35
SURB7
STK38L
FLJ22028
PPFIBP1
TM7SF3
>2
0
>2
Cluster 1
Cluster 2
Cluster 3
ARMC6
RQCD1
LRRC14
PRO2949
CAD
RBM28
TRIM25
LYAR
SNRPD1
LRP8
CCNE1
TIMM8A
ECE2
BYSL
CDCA7
WDR4
C1orf131
TIMM17A
CGI-115
MRPL15
CGI-12
WDSOF1
CML66
GTPBP4

Figure 6


A
EGFR activation
B
CRYAB activation
C
KRAS activation
D
Multiple activations
EGF
TGFA
EREG
AREG
HB-EGF
BTC
NRG1
NRG2
NRG3
NRG4
EGFR
HER2
HER3
HER4
SHC1
SHC2
SHC3
GRB2
SOS1
PLCG1
PLCG2
PIK3R1
PIK3CA
PIP2
PIP3
DG
IP3
PTEN
HRAS
NRAS
KRAS
PRKCA
PRKCG
PRKCB1
PDK-1
AKT1
AKT3
mTOR
BRAF
CRAF
ARAF
CRYAB
MEK1
MEK2
ERK1
ERK2
>2
0
>2
KRAS Amplicon
CGI-141
RECQL
FLJ10292
STRAP
FLJ10637
MRPS35
SURB7
STK38L
FLJ22028
PPFIBP1
TM7SF3
Cluster 1
Cluster 2
Cluster 3
ARMC6
RQCD1
LRRC14
PRO2949
CAD
RBM28
TRIM25
LYAR
SNRPD1
LRP8
CCNE1
TIMM8A
ECE2
BYSL
CDCA7
WDR4
C1orf131
TIMM17A
CGI-115
MRPL15
CGI-12
WDSOF1
CML66
GTPBP4

Toxicogenomics 2006 Bi-annual Meeting Speaker Abstract

EGFR Signaling Pathways in Breast Cancer

Katherine A. Hoadley and Charles M. Perou

The EGFR pathway is a complex signaling cascade that regulates proliferation, apoptosis, differentiation, mobility, and adhesion. In breast cancer, the gene and protein expression of EGFR varies across the subtypes. EGFR protein is high in 50% of basal-like tumors, but is rarely observed in estrogen receptor (ER) positive tumors. This suggests that there might be differences among the breast tumors subtypes in their EGFR signaling patterns.

To examine this hypothesis, we determined the sensitivity to EGFR inhibitors using cell line models of basal-like and luminal breast cancers. The basal-like subtype was more sensitive to EGFR inhibitors compared to the luminal subtype. We examined EGFR activation signatures by observing gene expression after removal of EGFR inhibitors. The top 500 induced genes were then clustered on a human breast tumor data set and three distinct clusters of genes were identified. One cluster was high in a mixed group of tumors, a second cluster was high in almost all the basal-like tumors, and a third cluster was high in luminal, HER2+/ER-, and basal-like subtype. The three different clusters were applied to an independent test set and high expression of each cluster was found to predict poor outcomes. While only 50% of basal-like tumors show high protein expression of EGFR, greater than 95% of basal-like tumors showed high expression of at least one of the EGFR-activation clusters. Chi-square analysis found that high expression of many genes downstream of EGFR (like KRAS and CRYAB) was correlated with the basal-like subtype. This data suggests that there may be multiple mechanisms downstream of EGFR that lead to EGFR-independent signaling. Inhibition further down in the pathway, such as with MEK inhibitors, may be an effective targeted therapy for the basal-like subtype.

Katherine A. Hoadley

7309 Calibre Park Drive Apt 305
Durham, NC 27707
Ph. 919-360-0651
Fax. 919-966-3015
Email: hoadley@med.unc.edu

University of North Carolina
CB# 7295, RM# 12-020
102 Mason Farm Road
Chapel Hill, NC 27599
Ph. 919-843-5717

EDUCATION

2006 **Ph.D. in Genetics and Molecular Biology**, University of North Carolina at Chapel Hill, NC

2001 **B.S. in Biology and B.A. in Chemistry**, West Virginia Wesleyan College, Buckhannon, WV

RESEARCH EXPERIENCE

Graduate Research Assistant, University of North Carolina at Chapel Hill, Curriculum in Genetics and Molecular Biology, August 2001 – December 2006.
Research Advisor: Charles M. Perou

- Studied chemotherapeutic and general stress responses of luminal and basal-like breast cancers using cell lines models and *in vivo* responses for each subtype.
- Employed cytotoxicity assays to assess phenotypic response to chemotherapeutics in breast cell lines.
- Gene expression studies of breast cell responses to cholesterol and HER1 inhibitors with specific emphasis on identifying potential therapies for basal-like breast cancer.
- Gene expression and cytotoxicity studies to examine how combinations of biologic agents and chemotherapeutics interact. Used statistical tools to assess synergy/antagonism due to co-treatment.
- Use of shRNA to knockdown TP53 and BRCA1 to establish their role in response to chemotherapy in the basal-like subtype.

Biological Science Aid, Appalachian Fruit Research Station, Agriculture Research Service, United States Department of Agriculture, Bardane, WV
1995-2001 (summers and part time during the academic year)

- Worked for a horticulturist, microbiologist, and plant physiologist
- Laboratory activities include assisting in carbohydrate analyses, preparation of culture media and plant tissues for *in vitro* culture, DNA and protein extractions from plant tissue, SEM preparation, biological pest control experiments, cloning and sequencing. Assist in care of experimental orchard, greenhouse crops, and washing glassware.

Biology Laboratory Assistant, West Virginia Wesleyan College, Buckhannon, WV, 1997-2001

- Assisted in biology laboratory preparations and *in vitro* culture media preparation.

TEACHING EXPERIENCE

Teaching Assistant for Genetic Analysis I, University of North Carolina at Chapel Hill, Curriculum in Genetics and Molecular Biology, Fall 2002, Fall 2003.

- Taught recitations and graded homework.

Teaching Assistant for Principles of Biology and Microbiology, West Virginia Wesleyan College, Buckhannon, WV, Biology Department, May 1999 – August 2001.

- Assisted with laboratory sessions.

PEER-REVIEWED PUBLICATIONS

Troester MA, **Hoadley KA***, Sørlie T, Børresen-Dale AL, Lønning PE, Herbert BS, Shay JW, Kaufmann WK, and Perou CM. 2004. Cell-type specific responses to chemotherapeutics in breast cancer. *Cancer Research* 64: 4218-4226.

Troester MA, **Hoadley KA***, Parker JS, and Perou CM. 2004. Prediction of toxicant-specific gene expression signatures following chemotherapeutics treatment of breast cell lines. *Environmental Health Perspectives Toxicogenomics* 112(16): 1607-1613.

Troester MA, Herschokowitz JI, Oh DS, He X, **Hoadley KA**, Barbier CS, and Perou CM. 2006. Gene expression patterns associated with p53 status in breast cancer. *BMC Cancer* 6:276

*Co-first author publications.

MANUSCRIPTS SUBMITTED

Penland SK, Keku TO, Torrice C, He X, Krishnamurthy J, **Hoadley Ka**, Woosley JT, Thomas N, Perou CM, Sandler R, and Sharpless NE. RNA expression analysis of formalin-fixed paraffin embedded tumors. Submitted *Lab Investigation*

Hoadley KA, Weigman VJ, Fan C, Sawyer LR, He X, Troester MA, Sartor CI, Rieger-House T, Bernard PS, Carey LA, and Perou CM. EGFR Signaling Varies with Breast Tumor Subtype. Submitted *Breast Cancer Research*.

FUNDED GRANT PROPOSALS

Development of Biologically Based Therapies for Basal-Like Tumors funded through Department of Defense Breast Cancer Research Program, Predoctoral Fellowship, March 2005 – Current.

HONORS/AWARDS

2006	Lineberger Comphrensive Cancer Center Retreat, 1st place Clinical/Translational Poster
2005	Toxicogenomics Research Consortium Student Poster Award
2005	UNC Graduate Student Kenan Hobgood Award
2003	International Congress of Genetics Travel Scholarship
2003	University of North Carolina Graduate School Travel Scholarship
2000-2001	Phi Kappa Phi, National Honor Society
1998-2001	Beta Beta Beta, National Biology Honorary
1999, 2000	West Virginia Wesleyan Outstanding Biology Student
1998-2001	Mrs. Alice S. Hill Designated Scholar
1997-2001	West Virginia Wesleyan Honors Program
1997-2001	West Virginia Wesleyan Presidential Scholar

PRESENTATIONS

EGFR Signaling Pathways in Breast Cancer. Empowering Environmental Heath Sciences Research with New Technologies: A Conference on Omics Applications in the Environmental Health Sciences. Chapel Hill, NC, December 4-6, 2006.

POSTER PRESENTATIONS

Hoadley KA and Perou CM. Identification of targeted therapies for basal-like breast tumors. European Breast Cancer Conference, Nice, France, March 21-25, 2006.

Hoadley KA and Perou CM. Identification of new therapies for basal-like breast tumors. National Institutes of Environmental Health Sciences Toxicogenomics Research Consortium Bi-Annual Meeting, Welches, OR, Dec 4-8, 2005.

Hoadley KA and Perou CM. Identification of drug targets for the treatment of basal-like tumors. Molecular Biology of Breast Cancer, Molde, Norway, June 21-26, 2005

Hoadley KA, Troester MA, and Perou CM. Tumor subtype specific chemotherapeutics responses in breast cancer. The Society of Toxicology, New Orleans, Louisiana, March 6-10, 2005

Hoadley KA, Troester MA, and Perou CM. Gene expression profiles of human breast cell lines treated with chemotherapeutics. The International Congress of Genetics, Melbourne, Australia, July 6-12, 2003